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A novel thermogelling matrix for microchannel DNA sequencing based on poly-*N*-alkoxyalkylacrylamide copolymers

We have developed a novel class of thermogelling polymer networks based on poly-*N*-alkoxyalkylacrylamides, and demonstrated their use as DNA sequencing matrices for high-throughput microchannel electrophoresis in capillary arrays. Polymers and copolymers of *N*-ethoxyethylacrylamide (NEEA) and *N*-methoxyethylacrylamide (NMEA) were synthesized by aqueous-phase free-radical polymerization and characterized by tandem gel permeation chromatography-multi-angle laser light scattering. These copolymer matrices exhibit "re-entrant"-type volume phase transitions, forming entangled networks with high shear viscosity at low (< 20°C) and high (> 35°C) temperatures, and undergoing a "coil-to-globular", lower critical solution temperature (LCST)-like phase transition over an intermediate temperature range (20–35°C). Hence, matrix viscosity is relatively low at room temperature (25°C), and increases rapidly above 35°C. The material properties and phase behavior of these thermogelling polymer networks were studied by steady-shear rheometry. These matrices are easily loaded into capillary arrays at room temperature while existing as viscous fluids, but thermogel above 35°C to form transparent hydrogels *via* a thermo-associative phase transition. The extent of the intermediate viscosity drop and the final viscosity increase depends on the composition of the copolymers. DNA sequencing by capillary array electrophoresis with four-color laser-induced fluorescence (LIF) detection shows that these thermogelling networks provide enhanced resolution of both small and large DNA sequencing fragments and longer sequencing read lengths, in comparison to appropriate control (closely related, nonthermogelling) polymer networks. In particular, a copolymer comprised of 90% w/w NMEA and 10% w/w NEEA, with a molecular mass of ~ 2 MDa, delivers around 600 bases at 98.5% base-calling accuracy in 100 min of electrophoresis.

Keywords: DNA sequencing / Microchannel electrophoresis / Thermogelling polymer network
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1 Introduction

The recent announcement of a completed, high-quality, comprehensive sequence of the human genome opens the door to a new era of genomic and biomedical research [1]. As continued efforts on high-throughput genome analysis are important for the advancement of basic science, human health, and society [2], a large amount of high-quality genetic information will be required and innovations in the development of robust, low-cost genetic analysis technologies are necessary. One of the key contributions to the field of genetic analysis has been the development of sophisticated microfluidic devices for DNA sequencing [3, 4]. Genome analysis on microfluidic devices promises to lead to substantial decreases in cost and analysis time, and offers the possibility of process integration to achieve sample preparation, purification, labeling, separation, and detection on a single platform [5–8].

In the early days of genome research, when slab-gel electrophoresis was the main workhorse technology, crosslinked polyacrylamide and agarose gels were used as the sieving matrices for DNA fragments. The transformation from a slab-gel format to capillary and subsequently to microchip (microchannel) formats has led to intense re-

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Abbreviations: GPC, gel permeation chromatography; **LCST**, lower critical solution temperature; **LPA**, linear polyacrylamide; **MALLS**, multi-angle laser light scattering; **NEEA**, *N*-ethoxyethylacrylamide; **NMEA**, *N*-methoxyethylacrylamide; **TTE**, Tris-TAPS-EDTA buffer

search in the development of matrices suitable for miniaturized systems. The use of linear polymer solutions for microchannel electrophoresis of DNA has proven to be an effective alternative approach, and a number of water-soluble, linear polymers including linear polyacrylamide (LPA) [9–12], poly-*N,N'*-dimethylacrylamide (pDMA) [13–15], and polyethylene oxide (pEO) [16], have been developed as DNA sequencing matrices. The characteristics, properties and performance of different sieving matrices have been reviewed recently [17, 18]. The major practical drawback of using replaceable, linear polymer solutions for microchannel electrophoresis is that while highly entangled solutions of high-molar mass polymers have proven to be most effective for high-resolution, long-read DNA sequencing separations [17, 19], the high viscosity of these polymer solutions requires the application of high pressure (*e.g.*, 1000 psi or greater) for matrix loading into microchannels. The selectivity of DNA separation in some cases may also be compromised by the dynamic and deformable properties of uncross-linked polymer networks. Moreover, the purchase of sieving matrix to "feed" capillary array electrophoresis instruments presently accounts for a substantial portion ($>$ 30%) of the total variable costs of high-throughput sequencing projects [20]. The further development and commercialization of microfabricated DNA sequencing devices is critical to bring costs down, but requires attention to matrix development to address the inherent difficulty of applying positive pressure to chip microchannels for matrix loading. Chip devices cannot physically withstand high delivery pressures ($\sim$ 200 psi is the highest pressure glass devices can typically withstand as high pressure will destroy the bonding between the cover and the substrate).

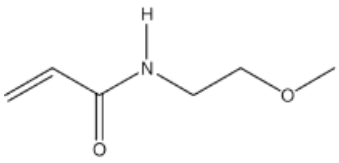
The development of polymeric matrices with "switchable viscosity" [21–26] is one strategy to decouple the capillary loading and DNA separation properties, which opens the door to the possibility of using glass/plastic microchips for high-throughput DNA sequencing [21]. For example, "thermothinning" polymer networks undergo a thermodynamically driven volume-phase transition [27], accompanied by a dramatic decrease in viscosity, in response to a change in temperature over a narrow range. The temperature at which this phase transition occurs is termed the lower critical solution temperature (LCST) or the "cloud point" of the solution, and is characterized by a sharp increase in turbidity of the polymer solutions [28]. Poly-*N*-isopropylacrylamide (pNIPA), with an LCST in water of 32°C, and hydroxypropylcellulose (HPC), with an LCST in water of 39°C, have been used as thermoresponsive sieving matrices for double-stranded (ds) DNA separations [29, 30]. Thermothinning polymer networks with designed LCSTs, based on linear copolymers of *N,N'*-dimethylacrylamide (DMA) and *N,N'*-diethylacrylamide (DEA), have been formulated as DNA sequencing matrices with a thermally controlled "viscosity switch" [22]. In particular, a copolymer composed of 42% w/w DEA and 58% w/w DMA delivered 575 bases in 94 min with a base-calling accuracy of 98.5% [21]. This copolymer network exhibits a dramatic drop in viscosity, of more than an order of magnitude, when heated above 80°C, which allows rapid matrix loading into the capillary lumen under very low applied pressure (50 psi). Upon reducing the temperature to below the LCST (to the sequencing temperature of 44°C), the entangled state of the polymer coils in solution is restored as they redissolve in aqueous solution, providing effective DNA sequencing performance.

Another interesting class of polymer matrices shows "thermothickening" behavior: these polymer networks exhibit an upper critical solution temperature (UCST) at which an expansion of coil volume occurs, accompanied by thermo-association of polymer chains and a dramatic increase in viscosity. Thermogelation is thus actuated with an increase in temperature. The advantage of thermogelling networks is that they can be designed to allow microchannel loading at room temperature, and then heated to the sequencing temperature to gel. A number of thermothickening polymer matrices have been developed based on polymers that exhibit thermo-associative behavior, with novel copolymer architectures such as poly-*N*-isopropylacrylamide-graft-polyethylene oxide (pNIPA-*g*-pEO) [30], poly-*N*-isopropylacrylamide-graft-polyacrylamide (pNIPA-*g*-LPA) [26], and pEO-polypropylene oxide block copolymers (pEO-pPO-pEO) [31]. These polymers utilize the self-associating properties of the hydrophobic chain parts, which serve as physical cross-linking points to form extended polymer networks when heated above the transition temperature. While it has been shown that these thermothickening polymer matrices can provide high-resolution dsDNA separations, single-base resolution of ssDNA under denaturing conditions (7 M urea, high temperature), as required for DNA sequencing, has not yet been presented in the literature for a thermogelling matrix.

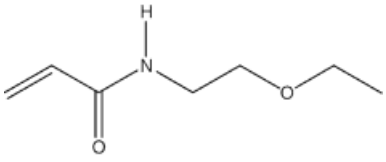
In this study, we report the synthesis and characterization of a novel class of thermogelling DNA sequencing matrices based on poly-*N*-alkoxyalkylamides, for application in capillary and chip electrophoresis. Poly-*N*-ethoxyethylacrylamide (pNEEA) hydrogels were first studied by Wada *et al.* in 1992 [32]. In the study, a cross-linked pNEEA gel disk was synthesized, and its swelling ratio was studied as a function of temperature in water. A "re-entrant"-type thermal response, *i.e.*, a solvent-swollen state at low ($<$ 25°C) and high ($>$ 40°C) temperature, and a shrunken (solvent-expelling) state at intermediate temperatures ($\sim$ 25–40°C), was observed for this cross-linked hydrogel

material. Based on this finding, we hypothesized that, at low temperature, a semidilute, uncross-linked solution of pNEEA should form a solvent-swollen entangled network, characterized by high steady-shear viscosity. A volume phase transition should then occur as polymer coils shrink by expelling solvent at increased temperature, and a drop in steady-shear viscosity would be expected to accompany this transition [22]. A further increase in temperature should then lead again to a swollen state, and hence the reformation of a robust entangled polymer network.

However, preliminary results showed that while linear pNEEA does exhibit the desirable re-entrant rheological behavior (even in a 7 M urea solution), the hydrophobic nature of the polymer prohibits DNA sequencing beyond ~100 bases, and the increase in turbidity accompanying the final phase transition disallows LIF detection. To address these problems, we investigated copolymers of NEEA and *N*-methoxyethylacrylamide (NMEA), a more hydrophilic monomer with similar structure, as a way to modulate and tune the rheological behavior, hydrophobicity, and optical properties of the polymer networks. The structures of NMEA and NEEA are shown in Fig. 1. We compared the properties and performance of NMEA/NEEA copolymers with those of polymers comprised of 100% NMEA, as well as 100% NEEA.



N-methoxyethylacrylamide (NMEA)



N-ethoxyethylacrylamide (NEEA)

Figure 1. Chemical structure of the NMEA and NEEA monomers.

2 Materials and methods

2.1 Polymer synthesis

Ultrapure (> 99.5% pure) NMEA and NEEA (Monomer-Polymer and Dajac Labs, Feasterville, PA, USA) were polymerized and copolymerized at different monomer ratios in an aqueous solution (1% w/v total monomer concentration), thermostated at 25°C, and degassed with nitrogen prior to initiation. The reactions were initiated with 0.5 μL per mL of monomer solution of a 10% w/v ammonium persulfate (APS) solution in water and 0.1 μL per mL of monomer solution of *N, N, N', N'*-tetramethylethylenediamine (TEMED) (both from Amresco, Solon, OH, USA). After 24 h, the resulting mixtures were removed from the water bath, poured into 100 000 molecular weight cut-off cellulose ester membranes (Fisher Scientific, Pittsburgh, PA, USA), and dialyzed against deionized, distilled water for 10 days with frequent water changes. The polymer solutions were then frozen and lyophilized using a freeze-drying system (Labonco, Kansas City, MO, USA), resulting in a stiff, white, foam-like polymer mass that was then redissolved in aqueous buffer by slow rotation overnight (Roto-Torque; Cole-Parmer Instrument Co., Vernon Hills, IL, USA).

2.2 Polymer molar mass distribution

The molar mass distributions of the NMEA polymer and NMEA/NEEA copolymers were determined by first fractionating the polymer samples by gel permeation chromatography (GPC) prior to analysis by on-line multi-angle laser light scattering (MALLS) and refractive index detection [33], using a Waters 2690 Alliance Separations Module (Milford, MA, USA) with Shodex (New York, NY, USA) OHpak columns SB-806 HQ, SB-804 HQ, and SB-802.5 HQ connected in series. In this tandem GPC-MALLS mode, the effluent from the GPC systems flows into the DAWN DSP laser photometer and Optilab interferometric refractometer (both, Wyatt Technology, Santa Barbara, CA, USA). Sample aliquots of 100 μL (sample concentration, 0.5 mg/mL) were injected into the system (mobile phase 0.1 M NaCl, 50 mM NaH_2PO_4, and 200 ppm NaN_3; flow rate, 0.30 mL/min). The tandem GPC-MALLS data were processed with ASTRA for Windows software from Wyatt Technology.

2.3 Rheological characterization

The steady-shear and temperature-dependent viscosities of the polymer solutions were measured with a Paar Physica MCR rheometer (Ashland, VA, USA) equipped with a cone-plate geometry (diameter, 25 mm; angle, 2°). The polymers were dissolved in sequencing buffer consisting of 50 mM tris(hydroxymethyl)aminomethane (Tris), 50 mM *N*-tris(hydroxymethyl)methyl-3-aminopropane-sulfonic acid (TAPS), and 2 mM ethylenediaminetetraacetic acid (EDTA) (0.5 × TTE, Amresco, Solon, OH, and Sigma, St. Louis, MO, USA) containing 7 M urea (Amresco) (0.5 × TTE, 7 M urea, pH 8), at the same polymer concentrations

used in the DNA sequencing experiments. Steady-shear viscosity at different rates of applied shear (from 0.01 s^{-1} to 1000 s^{-1}) was measured. Temperature-dependent viscosity was measured at a constant shear rate of 1 s^{-1} and at a heating rate of ~2°C/min.

2.4 DNA sequencing

DNA sequencing was performed on a MegaBACE 1000™ capillary array electrophoresis (CAE) system (Amersham Biosciences, Sunnyvale, CA, USA), equipped with four-color laser-induced fluorescence detection and 96 fused-silica capillaries (6 arrays of 16 capillaries with 75 μm inner diameter, 64 cm total length, 40 cm effective length, covalently coated with linear polyacrylamide). A 0.5 × TTE, 7 M urea buffer was used to dissolve the matrix polymers to desired concentrations. The DNA samples used were aliquots of the MegaBACE DNA sequencing standard (Amersham Pharmacia, Piscataway, NJ, USA) consisting of M13 DNA sequencing reaction products fluoresecently labeled with Amersham ET dyes. Sequencing matrix was loaded into the capillaries under an applied pressure of 1000 psi for 200 s, followed by a polymer relaxation time of 20 min and a prerun electrophoresis for 5 min at 140 V/cm and 44°C. After electrokinetic sample injection (46 V/cm, 40 s), the DNA was electrophoresed at 140 V/cm and 44°C (the highest temperature achievable for our instrument). Four-color laser-induced fluorescence data were collected, analyzed, and translated into DNA sequence using the MegaBACE 1000™ DNA sequencing software Version 2.0.

3 Results and discussion

3.1 Polymer molar mass characterization

Polymers synthesized for this study were characterized by tandem GPC-MALLS to determine the weight-average molar mass (M_w), weight-average radius of gyration (R_g), and polydispersity index (PDI) of each sample [33]. Figure 2 illustrates the molar mass distributions of four polymer samples, including 100% NMEA (pNMEA), 100% NEEA (pNEEA), 75% w/w NMEA/25% w/w NEEA (pNEEA25), and 90% w/w NMEA/10% w/w NEEA (pNEEA10). The physical properties of these polymers and copolymers are summarized in Table 1. Molecular weights of the matrices are ~2 MDa and are well matched, to facilitate a good comparison of properties and performance.

Prior research has shown that matrices composed of high-molar mass polymers (M_w > 10 MDa) are best suited to provide ultralong DNA sequencing read lengths (1000 bases or more in 1–2 h) [10, 19]. More "typical" read lengths of 500–700 bases can be obtained with linear polymers with M_w ranging from 2 to 5 MDa. An advantage of matrices based on lower-M_w polymers is their lower viscosities, which make capillary loading easier. Nonetheless, with hopes for long read lengths, free-radical solution polymerization conditions were adjusted to produce NMEA/NEEA copolymers with the highest achievable molar mass. We found that both NMEA and NEEA are vulnerable to cross-linking during polymerization at high monomer concentrations (> 5% w/v) and/or at high temperatures (> 40°C). However, with careful control of polymerization conditions (lower temperature (25°C) and with lower monomer concentrations (1% w/v), linear

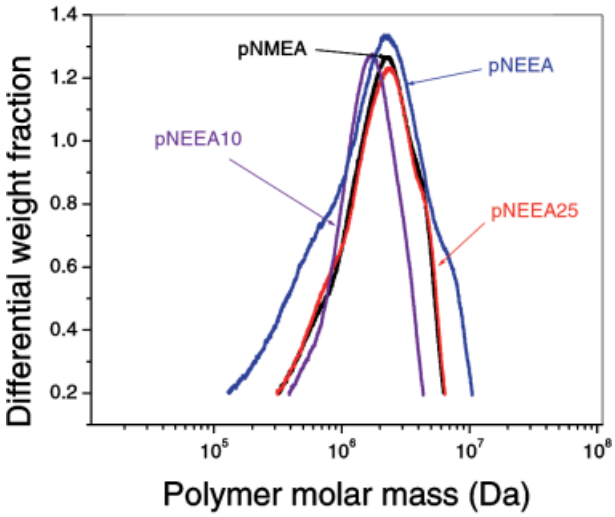


Figure 2. Molar mass distributions of pNEEA (blue), pNMEA (black), pNEEA25 (red), and pNEEA10 (purple) synthesized in this study, as characterized by tandem GPC-MALLS.

Table 1. Physical properties of NMEA and NEEA polymers and copolymers

Polymer	Weight-average molar mass, M_w (MDa)[a)]	PDI[a)]	Measured R_g[a)] (nm)
100% NEEA (pNEEA)	2.38	3.12	70.2
100% NMEA (pNMEA)	2.16	1.92	117.8
75% NMEA/25% NEEA (pNEEA25)	2.17	1.97	106.8
90% NMEA/10% NEEA (pNEEA 10)	1.98	1.45	107.2

a) Data represent the average of the results from three analyses.

copolymers with $M_w \sim 2$–3 MDa were obtained. Synthesis of higher molar mass NMEA/NEEA copolymers by inverse emulsion polymerization was attempted, as has been illustrated for the case of LPA [19, 34], however, the hydrophobic character of the copolymers prevented us from recovering them from the emulsion by precipitation in organic solvent.

3.2 Rheological behavior of polymer matrices

The polymers pNEEA, pNMEA, pNEEA25, and pNEEA10 were each dissolved in DNA sequencing buffer (0.5× TTE, 7 M urea), and their temperature-dependent rheological behavior was studied. Figures 3a–d show viscosity as a function of temperature for these polymers, between 20°C and 55°C. All four of these polymer matrices show distinct thermoresponsive behaviors. The expected "re-entrant"-type phase transition behavior is observed for linear pNEEA (Fig. 3a), as with the cross-linked hydrogel counterpart reported in the literature [32]. The "shrinking" behavior at intermediate temperatures was attributed to weak hydrophobic interactions of the alkoxyalkyl groups, while at higher temperatures, thermal mixing was hypothesized to dominate over hydrophobic interactions, leading to the subsequent swelling. Remarkably, even in 7 M urea, transitions in phase occur at about the same temperatures observed for the cross-linked disk in pure water [32]. Between 20°C and 35°C, viscosity drops from 3000 to ~ 600 cP; then between 35°C and 45°C, viscosity jumps rapidly up to ~ 20 000 cP, and then climbs towards a plateau of ~ 60 000 cP as temperature is increased further. This is the "thermogelling" phase transition. On the other hand, pNMEA shows only very weak thermoresponsive behavior, and only when heated above ~ 45°C (Fig. 3b), with a minor upturn in viscosity that might indicate the start of a phase transition which would mature at higher temperature. For the purposes of this study, in which the sequencing performance of these matrices was compared at 44°C, we considered pNMEA to be nonthermoresponsive and used it as our "control" network, to compare the effects of thermogelling and nonthermogelling behavior on DNA separation.

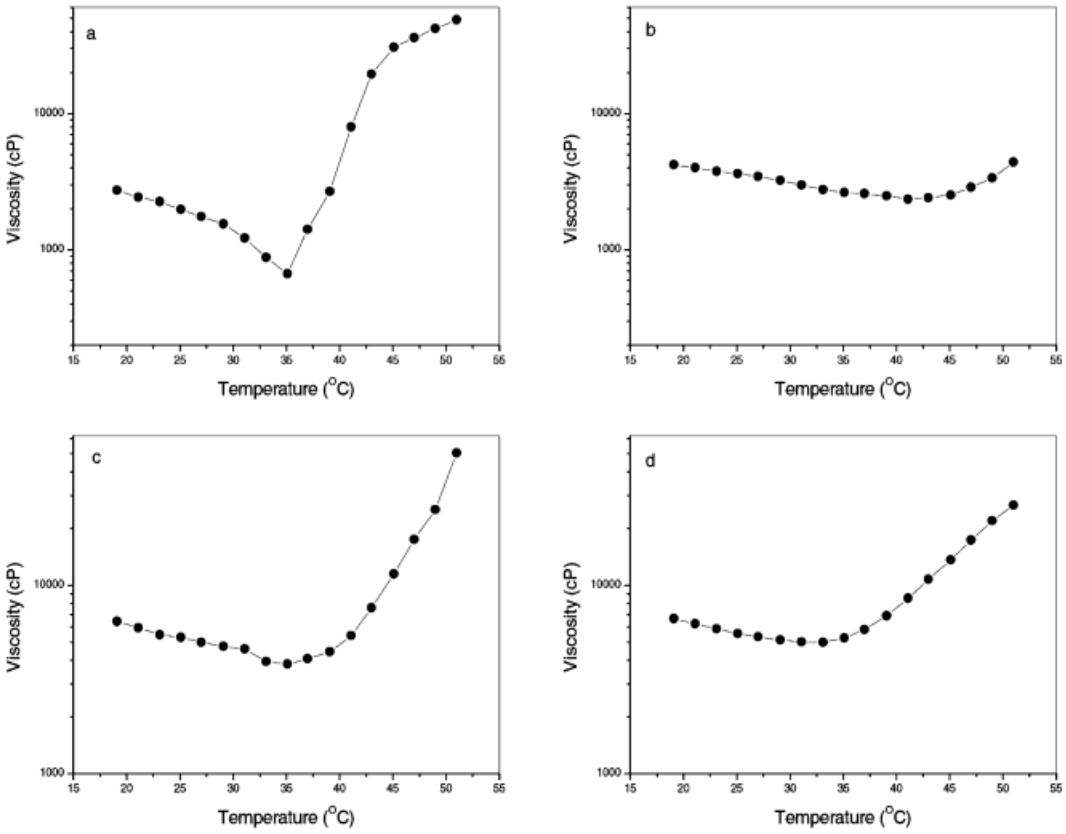


Figure 3. Temperature-dependent viscosities of (a) pNEEA, (b) pNMEA, (c) pNEEA25, and (d) pNEEA10 in 0.5× TTE/7 M urea solution at 7% w/v concentration. Experiments were performed with temperature control in a cone-and-plate fixture (diameter, 25 mm; angle, 2°) at a heating rate of ~ 2°C/min. Applied shear rate was 1 s^{-1}.

Copolymerizing NMEA and NEEA leads to changes in rheological behavior relative to either homopolymer, as expected. In comparison to Fig. 3a, the sharp drop in viscosity at ~35°C is modulated, and more gradual thermogelling occurs above this temperature. Thermogelling is more pronounced for pNEEA25 than for pNEEA10, as would be expected. It is interesting to note that phase transition behaviors of the copolymers occur at similar temperatures, regardless of monomer composition. The transition temperatures of both pNEEA25 and pNEEA10 are around 38°C (Figs. 3c–d). This observation differs from our earlier work that shows the volume phase transition temperature of thermoresponsive (LCST-exhibiting) polymers shifting in a monotonic fashion with changing copolymer composition [22]. This matter requires further study, which is presently ongoing in our laboratory.

3.3 DNA sequencing

Table 2 summarizes the DNA sequencing read lengths which were achieved in 100-min electrophoretic separations with 98.5% base-calling accuracy, using the polymers and copolymers of NMEA and NEEA as sieving matrices. A representative electropherogram obtained with pNEEA10 is presented in Fig. 4. As discussed above, the hydrophobicity of pNEEA is too high, which is found to prohibit effective DNA separation [35]. Also, the increased turbidity that occurs at the "re-entrant" phase transition of pure pNEEA disallows sensitive detection of fluorescently labeled DNA molecules in this matrix. Hence, the read length achieved with pNEEA was generally less than 100 bases (data not shown). On the other hand, a polymer matrix based on 100% NMEA, a more hydrophilic net-

Table 2. DNA sequencing read length obtained in different matrices

Polymer	Read length at (98.5% accuracy)
100% NEEA (pNEEA)	< 100
100% NMEA (pNMEA)	450
75% NMEA/25% NEEA (pNEEA25)	490
90% NMEA/10% NEEA (pNEEA10)	600

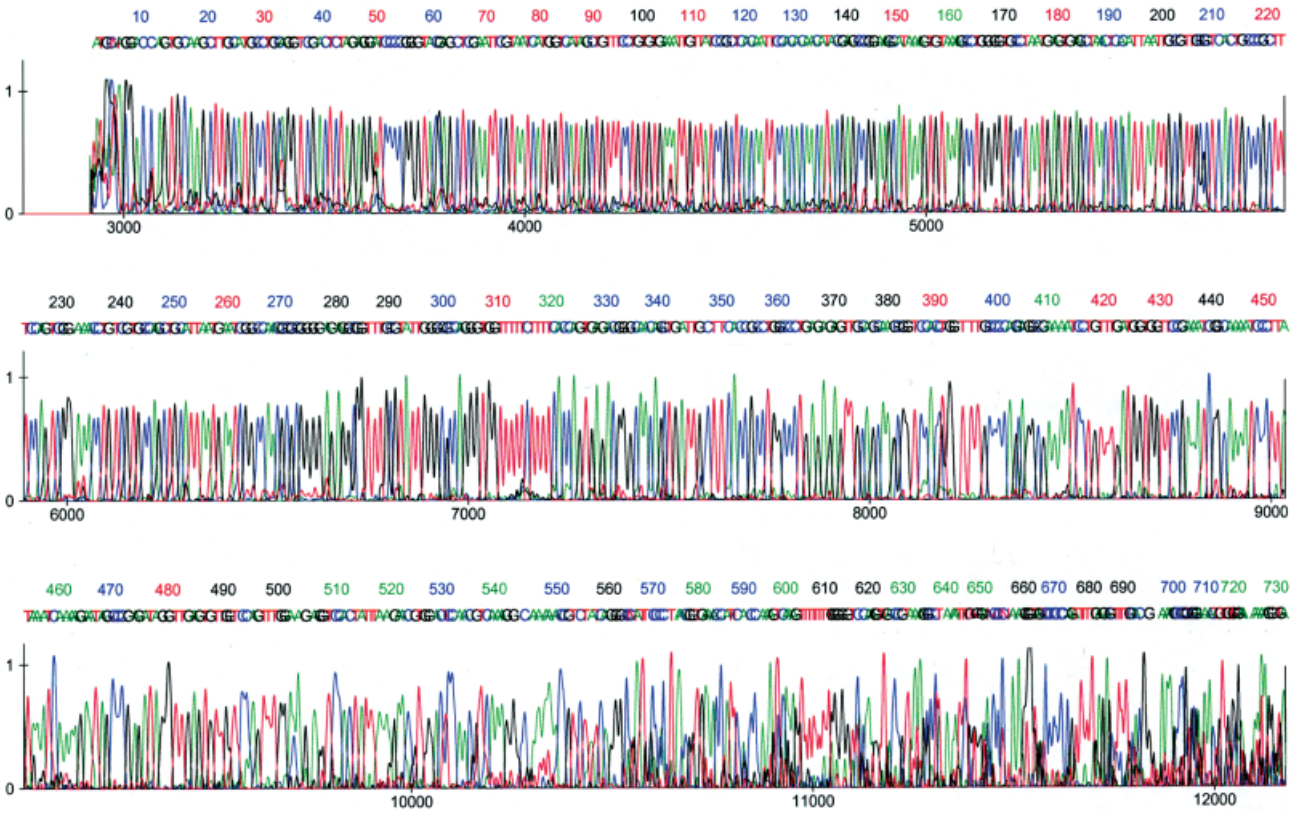


Figure 4. DNA sequencing electropherogram showing the separation of M13mp18 MegaBACE DNA sequencing standards using the pNEEA10 copolymer solution as the separation matrix. Separation was achieved in 7% w/v copolymer in 50 mM Tris/50 mM TAPS/2 mM EDTA buffer with 7 M urea, at 140 V/cm and 44°C. Other protocols as described in Section 2.4.

work, provides good sequencing performance with a relatively short read length, of 450 bases at 98.5% accuracy. We, therefore, explored copolymers of NMEA and NEEA in hopes of keeping to a minimum the hydrophobicity of the matrices but, at the same time, harnessing the thermoresponsive behavior of the NEEA comonomer. We found that copolymers of NMEA and NEEA provide good DNA sequencing performance, even better than that of pNMEA homopolymers: pNEEA25 gave a 490-base read, and pNEEA10 gave a 600-base read, both at 98.5% base-calling accuracy. We believe that the improved performance of the copolymers relative to the more hydrophilic pNMEA is due to the thermogelling behavior of the copolymers, which provides higher resolution *via* physical stabilization of the polymer networks. Hence, the copolymer networks combine the fluid behavior of a linear, entangled polymer solution at room temperature with the static, nondeformable nature of cross-linked hydrogels at sequencing temperatures, and provide a significantly extended read length relative to what can be achieved in pure, nonthermogelling pNMEA. However, because pNEEA exhibits substantial hydrophobicity at high temperature, a lower amount of NEEA incorporated into the copolymer (10% *vs.* 25%) is clearly beneficial to DNA separation. We conclude that an optimal copolymer formulation must include a sufficient fraction of the NEEA monomer to give the thermogelling behavior, while the hydrophobicity should also be minimized by keeping the fraction of NEEA as low as possible. Further optimization of the polymer molar mass, molar mass distribution and composition could potentially lead to matrix performance improvements. Note that the zero-shear loading viscosity of the NMEA/NEEA copolymer matrix is lower than that of high-molar mass LPA by more than one order of magnitude (data not shown); this presents a distinct advantage of the thermogelling polymer networks, especially for application in microfluidic devices. On the other hand, the performance is not quite as good as what would be obtained in LPA matrix with respect to read length (based on experiments performed in our laboratory using commercial matrices (Beckman LongRead®) under the exact same conditions). As usual it turns out to be a trade-off between loading viscosity and read length.

There are other, possible routes to improving the performance of these thermogelling networks. As shown in the temperature-dependent viscosity data, the transition temperature of the pNEEA10 copolymer solution is close to 40°C. While further temperature increase showed no sign of creating a viscosity plateau, we hypothesize that with temperature $>$ 44°C a more strongly associated polymer network would be formed, and hence better DNA sieving could be achieved at a sequencing temperature of 50°C or beyond. It has been shown that the optimal DNA sequencing temperature lies between 50°C and 60°C [36]. However, the highest achievable sequencing temperature of the MegaBACE 1000™ system in our laboratory is 44°C, and we believe this limits our ability to observe the full potential of these thermogelling matrices. Finally, base-calling accuracy could be improved substantially with custom mobility shift corrections for the four different, base-specific dyes, as the base-caller we used is "trained" to account for matrix-specific mobility shifts for LPA, which is a very hydrophilic polymer with chemical structure very different from the poly-*N*-alkoxyalkylacrylamide network.

The DNA sequencing performances of different polymer matrices can be quantified to identify matrix-specific factors that limit read length, and to provide guidance towards the formulation of optimal matrix and CE conditions. Plots of migration time *vs.* base number for the different polymer matrices were derived from the electropherograms, and fitted with a third-order polynomial [34]. The polynomial functions were then used to calculate the selectivity of DNA separation ($\Delta\mu/\mu_{avg}$) according to the following equation:

$$\left|\frac{\Delta\mu}{\mu_{avg}}\right| = 2\left|\frac{t_{m1} - t_{m2}}{t_{m1} + t_{m2}}\right| \quad (1)$$

where t_m is the migration time of the DNA sequencing peak of interest. Figure 5 shows plots of selectivity *vs*. DNA base number for the three interesting polymer matrices (excluding pNEEA which gave $<$ 100 bases). Selectivity for small-base number DNA was higher than that for large-base number DNA for each matrix, decreasing with DNA size with a roughly linear dependence. The

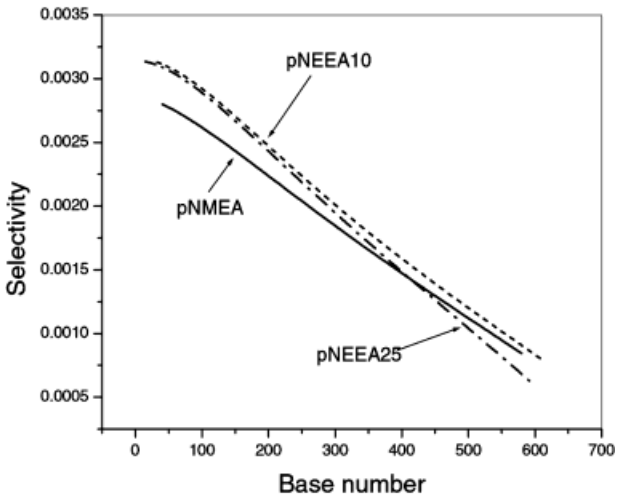


Figure 5. Selectivity *vs.* base number for pNMEA (— —); pNEEA25 (–·–·); and pNEEA10 (– – –) matrices, for the CAE separation of M13mp18 MegaBACE DNA sequencing standards. Separation conditions as in Fig. 4.

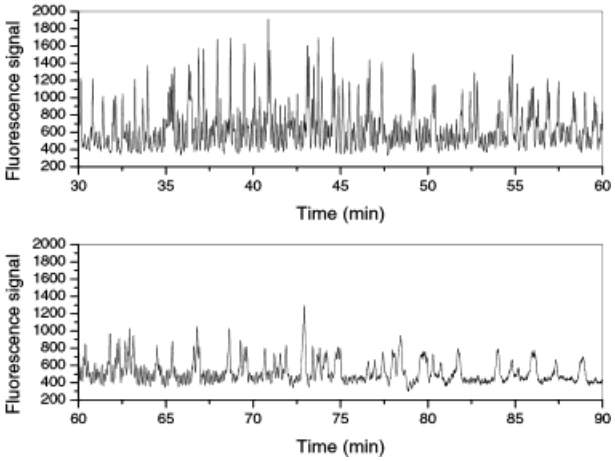


Figure 6. Raw fluorescence signal for the T-terminated sequence of M13mp18 Sanger DNA fragments, obtained with the pNEEA10 copolymer solution as the separation matrix. Separation conditions as in Fig. 4.

selectivity of the pNEEA10 copolymer matrix is generally highest, as is consistent with our anticipation based on the balance of retaining thermogelling behavior with the minimum polymer hydrophobicity. The selectivity of the pNEEA25 matrix is lower than that of pNMEA for DNA larger than 400 bases, probably due to the hydrophobic effect. The pNEEA10 matrix, however, performs better than pNMEA at high base numbers, probably in this case because of the thermogelation. The significantly higher selectivity we observe for small DNA fragments in both copolymer networks suggests that other than high-throughput sequencing, which usually requires high resolution of larger DNA fragments (*i.e.*, $>$ 500 bases), these thermogelling polymer networks should be ideal for other genomic analyses such as minisequencing or dsDNA analysis for PCR fragment sizing or microsatellite analysis. It is important to note that no significant drop in the slopes of these selectivity plots was observed for any of the three matrices as a function of DNA size, which indicates that the read length is not limited by diminishing selectivity, as would be reflected by a plateau of migration time, which we do not observe (data not shown). The absence of a migration time plateau suggests that biased reptation is not occurring in these matrices over this DNA size range; therefore, a longer read length may be obtainable with the optimization of other parameters. We also analyzed the effects of the thermogelling behavior on DNA peak width; however, no strong trends or major differences in the three matrices were observed.

A careful examination of the raw fluorescence signal enables us to investigate some potential effects of the chemical structures of the polymer matrices on sequencing performance. Figure 6 shows the raw fluorescence signal of the T-terminated sequence of M13mp18 DNA sequencing standards, obtained with pNEEA10. The relatively high background signal, or low signal-to-noise ratio, which we observe is probably a major limitation to obtaining longer read length. Several factors can lead to low signal-to-noise ratio, including an insufficient amount of injected sample, and/or matrix turbidity resulting from the thermogelling phase transition. These and other possible sources of read length limitation will be investigated further.

4 Concluding remarks

We have designed, formulated, and tested a novel class of thermogelling polymeric sieving matrices based on poly-*N*-alkoxyalkylacrylamides. NMEA/NEEA polymers and copolymers with average molecular weight $\sim$2 MDa were synthesized by aqueous-phase reaction and characterized by tandem GPC-MALLS. We find that by copolymerizing NEEA with NMEA, we can control the overall hydrophilicity of the polymer matrices and at the same time harness the desired, thermoresponsive behavior of pNEEA. We observe a significant improvement in DNA sequencing read lengths in a thermogelling polymer network, relative to the performance of a nonthermogelling control. In particular, a copolymer composed of 90% w/w NMEA and 10% w/w NEEA delivers a 600-base read, about 150 more bases than the nonthermogelling pNMEA at 44°C. The results presented show that copolymers of NMEA and NEEA are promising candidates for novel DNA sequencing matrices for use in microfluidic devices. Further work will be devoted to optimizing the polymer molar mass and copolymer composition, understanding read length limitations, and testing thermogelling matrices for DNA sequencing on glass chips.

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